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Eubacterial proteasomes

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Abstract

Proteasomes are large, multisubunit proteases with highly conserved structures. The 26S proteasome of eukaryotes is an ATP-dependent enzyme of about 2 MDa, which acts as the central protease of the ubiquitin-dependent pathway of protein degradation. The core of the 26S complex is formed by the 20S proteasome, an ATP-independent, barrel-shaped protease of about 700 kDa, which has also been detected in archaebacteria and, more recently, in eubacteria. Currently, the distribution of 20S proteasomes in eubacteria appears limited to the actinomycetes, while most other eubacteria contain a related complex of simpler structure.

Introduction

The 26S proteasome is a large ATP-dependent proteolytic complex found in the cytosol and nucleus of all eukaryotic cells. It is an abundant protein, which represents the core enzyme of the non-lysosomal path of protein degradation and thus plays a key role in many cellular processes (reviewed in [1–3]).

The 26S complex consists of two asymmetric 19S caps flanking a barrel-shaped 20S core (the 20S proteasome) [4]. The 19S caps contain the ATPase function and select the targets for degradation, which they present in unfolded form to the 20S core. The caps consist of approximately 15 different subunits between 25 and 110 kDa, six of which are ATPases belonging to the AAA family [5–8].

The 20S core degrades the substrates to peptides of approximately 4–10 residues length [9, 10]. It consists of 14 different but related subunits of about 22–35 kDa, which can be divided into an α-type and a β-type group on the basis of their sequence similarity (Figure 1) [11, 12]. The subunits are arranged into four seven-membered rings [13, 14], with the α-type subunits forming the outer and the β-type subunits the inner rings of the complex [15–18]. Collectively, the four rings form an elongated cylinder of 15 nm length and 11 nm diameter [14, 19–21], which is traversed from end to end by a channel and contains three large inner cavities bounded by four narrow constrictions at the level of the α- and β-subunit rings [14, 22]. Substrates penetrate into the proteasome via the central channel; as expected from the dimensions of the α-ring constrictions, only entirely unfolded polypeptide chains can pass [23]. By a still unknown mechanism, substrates wind their way through the outer cavities to the proteolytic active-site clefts in the central cavity.

As anticipated from their sequence similarity, α- and β-type subunits have the same fold [14]: a four-layer $\alpha + \beta$ structure with two central antiparallel β-sheets, flanked on either side by α-helices. The β-sheet sandwich is open at one end to form the active-site cleft. The main difference between α- and β-type subunits lies in a highly conserved N-terminal extension of α-subunits, which forms an α-helix across the top of the central β-sandwich, thus filling the active-site cleft. Correspondingly, only the β-type subunits have proteolytic activity. The function of the N-terminal extension is not clear, but its location at the top of the α-rings close to the entrance of the channel indicates that it may be important for interactions between the

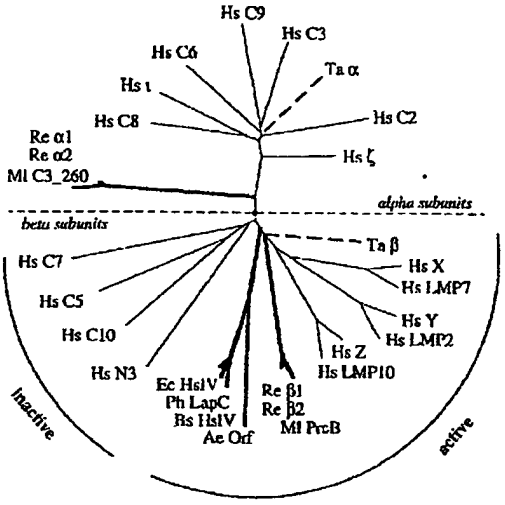

Figure 1. Dendrogram of 20S proteasome subunits, showing their classification into α-type and β-type sequences. Archaebacterial branches are dotted and eubacterial branches are bold. The organisms are: Hs, *Homo sapiens*; Ta, *Thermoplasma acidophilum*; Re, *Rhodococcus erythropolis*; Ml, *Mycobacterium leprae*; Ec, *Escherichia coli*; Bs, *Bacillus subtilis*; Ph, *Pasteurella haemolytica*; Ae, *Alcaligenes eutrophus*. The division into active and inactive β-type subunits was deduced from the conservation pattern of active-site residues [31] and from the location of the processing site (Figure 2). For the three branches of active human β-type subunits, the γ-interferon inducible alternates are also shown.

proteasome and its regulatory complexes, as well as for guidance of the unfolded substrates through the channel. In place of the N-terminal α-helix, β-type subunits contain a prosequence (Figure 2) [16, 24–28], which is cleaved off during proteasome assembly to free the active site [29, 30]. The cleavage occurs on the N-terminal side of a threonine residue, which may act as both the catalytic nucleophile and the primary proton acceptor in the mature subunit [14, 31–33]. Beside the N-terminal threonine, β subunits require two further residues for activity, whose exact role remains to be clarified: a lysine residue and a negatively charged residue, which form a salt bridge across the bottom of the active site and may participate in the delocalization of the threonine side-chain proton by forming a charge relay system [14, 31].

26S and 20S proteasomes are ubiquitous in eukaryotes. 20S (but not 26S) proteasomes have also been discovered in the archaebacteria *Thermoplasma acidophilum* [21], *Methanosarcina thermophila* [34], and (by genomic sequencing) in *Methanococcus jannaschii* [35]. These are composed of only one α and one β subunit and thus have a simpler structure than eukaryotic proteasomes, but are otherwise indistinguishable in their quaternary structure at low resolution (1.5 nm). Recently, 20S proteasomes [28] as well as simpler complexes corresponding to the two central proteasome rings [36] have been discovered in eubacteria.

Proteasome sequences in eubacteria

The first report of multicatalytic eubacterial proteases possibly related to the proteasome was made from the actinomycete *Frankia* strain BR [37]. Several proteolytic complexes between 270 kDa and 1.3 MDa were identified in crude extracts, but the low amount of material, which was due to the extremely slow growth of the organism, precluded the purification and sequence determination of these complexes.

The similarity between a eubacterial sequence and a proteasome subunit was first noted during the *Escherichia coli* genome project for the heat-shock locus V gene (*hslV*) [38]. A search of sequence databases revealed the existence of several eubacterial proteins related to proteasome β-type subunits [39]. These fell into two groups (Figure 1): the sequences from *E. coli*, *Pasteurella haemolytica*, *Bacillus subtilis* and *Alcaligenes eutrophus*, which are all HslV homologues and contain either no prosequence or only a short one, and the sequence from the actinomycete *Mycobacterium leprae*, which resembles eukaryotic and archaebacterial subunits more closely and contains a prosequence of 56 residues (Figure 2). The HslV homologues (but not the *M. leprae* sequence) were found to occur in an operon with an ATPase of the Clp family (Figure 3), suggesting that they form a hybrid structure combining features of the Clp protease with those of the proteasome. In contrast, the sequence of *M. leprae*, which has greater similarity to eukaryotic sequences and contains a large prosequence, was proposed to form a structure of the 20S type.

Independently, an operon with striking similarity to the *M. leprae* proteasome gene region was found during the sequencing of the *thc* gene cluster in the soil actinomycete *Rhodococcus erythropolis* NI86/21 [28, 40]. The similarity included a sequence identity of 61–81% on the protein level, and, on the DNA level, a translational coupling between the three genes in the operon. In addition to the proteasome β-type sequence, the operon was proposed to also contain a highly divergent α-type sequence, supporting the hypothesis that actinomycetes contain a proteasome of the 20S type.

archaebacterial β subunits

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Ta β                                                                MNQTLETGTTTVG
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eubacterial β subunits

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Rs β1          MTADRPALRTGDRDTRLSFGSNLSSFTDYLRGHAPELLPENRIGHRSHSTRGGDGMESGDLAPHGTTIVA
Rs β2          MTVDR-APRITDGDTRLSFGSNLSSFSEYLRVHAPEHLPQNRFADT-----GGVVMGGGDVAPHGTTIVA
Ml β           MTRSFPDRLPTNLAFPGISVINQSSFVDLLRRQAPELLPVSL---------GGGQSGGGQQLSHGTTIVV
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HslV family

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Ec HslV                                                                    MTTIVS
Ph LapC                                                                    MTTIVC
Ae Orf                                                                     MTTCVV
Bs HslV                                                                  MSFKGTTVIA
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eukaryotic β subunits

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Hs Z                                                  MAAVSVYAPPVGGFSFDNCRRNAVLEADFAKRGYKLPKVRKTGTTIAG
  Hs LMP10                                          MLKPALEPRGGFSFENCQRNASLERVLP--GLKVPSARKTGTTIAG
Hs Y                                                    MAATLLAARGAGPAPAWGPEAFTPDWESREVSTGTTIMA
  Hs LMP2                                                MLRAGAPTGDLPRAGEVETGTTIMA
Hs X                                                                  ...PEEPGIEMLEGTTTLA
  Hs LMP7  MALLDVCGAPRGQRFESALPVAGSGRRSDPGHYSFSMRSPELALPRGMQPTEFFQSLGGDGERMVQIEMAHGTTTLA

Hs N3                                  MEAPLGSRSGLWAGGPAPGQFYRIPSTPDSFMDPASALYRGPITRTQNPMVTGTSVLG
Hs C5                                                MLSSTAMYSAPGRDLGMEPHRAAGPLQLRPSPYVFNGGTILA
Hs C7                                                                          MEYLIG
Hs C10                                                                       MSIMSYNGGAVMA
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Figure 2. Prosequences of β-type proteasome subunits (bold). The sequence names are as in Figure 1.

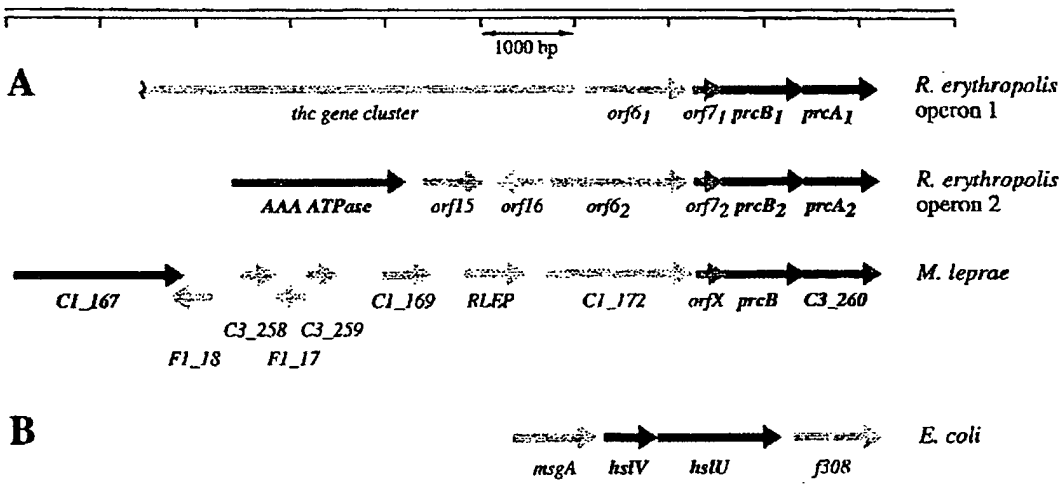

Figure 3. Operon structure of actinomycetal 20S subunits (A), and of the HslVU protease (B). β-type subunit genes (*prcB1, prcB2, prcB, hslV*), α-type subunit genes (*prcA1, prcA2, C3–260*), and ATPase genes (*AAA ATPase, C1–167, hslU*) are labeled in boldface. The *thc* genes, whose cluster extends beyond the region shown here, encode proteins required for thiocarbamate degradation [40]. RLEP is a copy of the *M. leprae*-specific dispersed repetitive sequence.

Because *M. leprae* is a is an obligately intracellular pathogen that cannot be cultivated *in vitro*, the subsequent effort to isolate a eubacterial proteasome was concentrated on *Rhodococcus*.

The proteasome of *Rhodococcus erythropolis*

Isolation and biochemical characterization

The proteolytic activity of 20S proteasomes is resistant to low amounts of SDS. This property was exploited

in order to identify the fraction of *Rhodococcus* cell extract containing the 20S proteasome [28]. The complex was then purified to more than 95% homogeneity by four successive chromatography steps. The substrate specificity of the particle as assayed with fluorigenic peptides was similar to that of the archaebacterial proteasome from *Thermoplasma*. High levels of activity were measured for most chymotryptic substrates, whereas tryptic and petidyl-glutamyl substrates were not measurably degraded. It should be noted though that this specificity has little (if any) relevance for the actual activity against protein substrates and thus for the *in vivo* situation, as was shown for the proteasomes from *Thermoplasma* and man [9, 10]. It is clear from the crystal structure of the *Thermoplasma* proteasome that a classification of specificities based purely on the residue at the P1 position must fall short of reality, given the multiple interactions the proteasome makes with residues up to position P4, and possibly beyond [14].

SDS-polyacrylamide gel electrophoresis of the *Rhodococcus* proteasome surprisingly yielded four different bands of similar intensity, rather than the two expected from the operon sequence [28]. Protein sequence analysis by Edman degradation showed that the two top bands corresponded to similar but non-identical α-type subunits and the two bottom bands to β-type subunits. Only one each of the α- and β-type subunits were encoded in the already known operon of proteasome genes, indicating the presence of a hitherto unknown second operon, which was identified by gene hybridization (Figure 3). The two operons are very similar (about 80% sequence identity on the protein level) and have obviously diverged much more recently that the operons of *Rhodococcus* and *Mycobacterium*, but have a different GC content. A screen of other *R. erythropolis* strains yielded in all cases only one operon corresponding to the second operon of strain NI86/21 (I. Nagy and R. De Mot, unpublished). This operon has a GC content of 63% while the first operon of strain NI86/21 has a significantly higher content (67%), indicating that it was acquired by horizontal transfer from a related actinomycete, rather than originating by gene duplication. The occurrence of only one operon of proteasome genes in most *R. erythropolis* strains indicates that four subunits are the exception rather than the rule in eubacterial proteasomes.

Quaternary structure

At the resolution of electron micrographs, the proteasome from *Rhodococcus* was indistinguishable from archaebacterial and eukaryotic proteasomes [28]. Negatively stained preparations showed the two characteristic views: ring-shaped end-on views of about 11 nm diameter and rectangular side-views of 15 nm length. Because it is notoriously difficult to analyze the symmetry of particles formed by several rings stacked out of register (especially as even small tilts of the cylinder axis cause severe distortions in the projection images), it was not possible to determine from image analysis of end-on views whether the rings are formed by six or seven subunits. Nevertheless, the similarity of the side-views to those of proteasomes from other organisms indicates that the rings are seven-membered. To decide this question and to determine the position of the different subunits in the complex, an expression system was set up in *E. coli*, in which the four subunits could be expressed in all possible combinations (F. Zühl and W. Baumeister, unpublished).

All four combinations of one α and one β subunit yielded fully assembled and proteolytically active complexes, which are indistinguishable from each other and from native proteasomes in electron micrographs (Figure 4). Obviously the subunits have not diverged to an extent that would prevent productive interaction. In all four cases as well as for the proteasome isolated from *Rhodococcus*, native gel electrophoresis yielded sharp single bands with an apparent molecular mass corresponding to complexes formed by seven-membered rings. This result strongly favors a (pseudo)sevenfold symmetry for the *Rhodococcus* proteasome. Mixtures of two recombinant proteasomes could be clearly separated by native gel electrophoresis, excluding the possibility that the *Rhodococcus* proteasome is in fact a mixture of two distinct complexes. This is supported by measurements of the K_m, which show the constants of the four recombinant proteasomes to be very different (and much lower) than the constant of the native complex (Figure 4). Such a result could not be obtained by simple mixtures of different complexes and indicates that the two β subunits interact in the native complex. Two subunit topologies remain possible which cannot be resolved at this point: stacks of four different homooligomeric rings or α and β rings with a random subunit distribution.

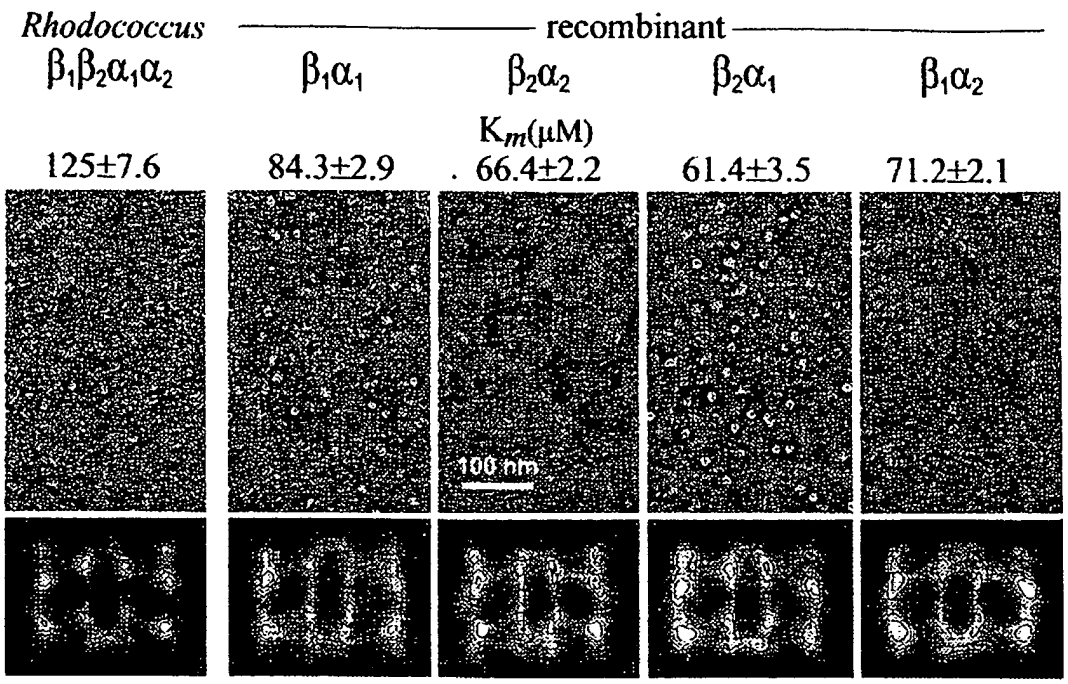

Figure 4. Electron micrographs of native and recombinant *Rhodococcus* proteasomes with the corresponding averages of the side views at the bottom. At the top are listed the respective K_M values.

Processing and assembly

Both β subunits of *Rhodococcus* are synthesized in an inactive precursor form and are activated by cleavage at an internal threonine residue during maturation of the proteasome [28]. When the β subunits are expressed without α subunits they remain unprocessed (and therefore inactive), as expected from experiments with the *Thermoplasma* proteasome. The fact that the β subunits are processed correctly in all four recombinant proteasomes described in the previous section shows that in *Rhodococcus*, both α subunits can support the processing of both β subunits.

Unlike *Thermoplasma*, where the α subunits assemble spontaneously into seven-membered rings [29], the α subunits of *Rhodococcus* are not capable of self-assembly either when expressed separately or jointly (F. Zühl and W. Baumeister, unpublished). Preliminary results also indicate that unlike in *Thermoplasma* but like in eukaryotic proteasomes [41, 42], the assembly proceeds *via* intermediate 'half-proteasomes'. This points to significant differences between the assembly pathways in *Thermoplasma* and *Rhodococcus*.

Is the Rhodococcus *proteasome of eukaryotic origin?*

Proteasomes have so far been detected in several actinomycetes but in no other eubacteria, and the genome sequences of *Haemophilus influenzae* [43] and *Mycoplasma genitalium* [44] show them to be absent from these organisms. It therefore seems conceivable that the actinomycetes have acquired the proteasome genes by horizontal gene transfer after their separation from other Gram-positive bacteria. Features of the assembly pathway, the sensitivity to inhibitors and the length of the β-type subunit prosequences all point to a greater similarity between the eubacterial and eukaryotic proteasomes, indicating that this transfer may have occurred from a eukaryotic organism.

In eukaryotes, the 20S proteasome interacts with the 19S caps, which contain a set of ATPases of the AAA family [8, 45–48]. Intriguingly, an ATPase of the AAA family is found in close proximity to the proteasome genes in *Rhodococcus* and *Mycobacteri-*

um. Although its sequence is too divergent to allow its assignment to the 19S group of ATPases, it contains several features indicating that it in fact belongs to this group: It has the same domain structure consisting of an N-terminal coiled coil, followed by a region of about 60 residues, and ending with the AAA ATPase domain, which extends to the C-terminus of the protein. Within AAA ATPases, N-terminal coiled-coil segments have so far only been found in the 19S group [12]. Also, both the *Rhodococcus* and the *Mycobacterium* ATPases contain a tyrosine as the one-but-last residue, a feature that appears to be characteristic for the 19S and CDC48/p97 groups of AAA ATPases. The ATPase from *Rhodococcus* has been cloned, sequenced, and expressed in *E. coli* (S. Wolf, R. De Mot, and W. Baumeister, unpublished). It has a low, N-ethyl maleimide-sensitive ATPase activity typical of AAA ATPases and forms a cylindrical complex of two six-membered rings. This complex forms and is stable in the absence of ATP. An interaction between this ATPase and the *Rhodococcus* proteasome is being explored but has not yet been demonstrated.

The HslV protease

Although most eubacteria do not appear to contain a 20S proteasome, they contain a related protease complex of simpler quaternary structure. This complex, HslV, is formed by only two stacked rings of β-type subunits, with an overall height of 8 nm and a diameter of 11 nm (M. Rohrwild, A. Goldberg, and W. Baumeister, unpublished). It essentially corresponds to the two central rings of the proteasome; however, HslV is built of six-membered rings.

The HslV protease occurs in an operon with an ATPase of the Clp family, HslU [38]. This arrangement of a protease with an ATPase in one operon parallels the operon structure of the ClpXP protease [49], which is also heat-shock inducible, except that here, a Clp-type ATPase is paired with a proteasome-type protease. Both HslU and HslV have been purified to apparent homogeneity [36, 50]. The HslU subunits can associate into six- and seven-membered rings in the presence of ATP or non-hydrolyzable analogues. The HslU rings can then bind to both ends of the HslV core protease. This association stimulates the proteolytic activity of HslV by two orders of magnitude in the presence of ATP but not in the presence of non-hydrolyzable analogues.

In their general architecture, the HslVU and Clp protease complexes resemble the 26S proteasome: the proteolytic activity is confined to the innermost cavity of a molecular microcompartment, well segregated from the cellular environment and guarded by ATPase rings which select and unfold the proteins targeted for degradation. It therefore seems likely that corresponding ATPase complexes will also be shown to exist for the *Rhodococcus* 20S proteasome.

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